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Sensitivity profile of triple-negative breast cancer cell lines to combination therapies including RANKL blockade

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RESUMO

O cancro da mama (CM) é o cancro com a segunda mais elevada mortalidade a nível mundial. Dada a heterogeneidade que apresenta, é classificado em subtipos de acordo com a expressão de determinados recetores moleculares, de modo a permitir uma maior especificidade, e consequentemente eficácia, no tratamento dos pacientes. Um dos subtipos definidos é o CM triplo negativo (TNBC, do inglês *triple--negative breast cancer*), caraterizado pela ausência de expressão de recetores de progesterona (RP) e de recetores de estrogénio (RE), e ainda por não apresentar amplificação do recetor 2 do fator de crescimento epidérmico humano (HER2).

O TNBC constitui cerca de 10% a 15% dos casos de CM e está associado a um prognóstico pior do que os restantes subtipos, possuindo os pacientes taxas de sobrevivência inferiores e maior probabilidade de recidiva. Apesar de o TNBC apresentar uma elevada sensibilidade à quimioterapia, sendo esta a terapia padrão para os doentes com TNBC, a aquisição de mecanismos de resistência explica as recidivas e pior prognóstico observados. Deste modo, é necessária a investigação de novas abordagens clínicas direcionadas que reduzam o impacto negativo do TNBC na sociedade.

Os inibidores da poli-(ADP-ribose) polimerase (PARP) (iPARP) são uma classe de fármacos que afeta a reparação de quebras simples da cadeia de DNA (SSB, do inglês *single strand breaks*). Em paralelo, CM com mutações nos genes *BRCA1* e/ou *BRCA2* tendem a perder o alelo saudável, o que lhes confere deficiência na reparação por recombinação homóloga (HRR, do inglês *homologous recombination repair*), impedindo a correção de quebras duplas da cadeia de DNA (DSB, do inglês *double strand breaks*). Estes dois défices resultam eventualmente em apoptose. O olaparib é um iPARP aprovado para tratamento de CM, nomeadamente em doentes com CM $HER2^-$ metastático ou avançado com mutações germinais em *BRCA1/2* e de doentes com CM com recetores hormonais (RH) não responsivos a terapia hormonal (TH). É possível que outros doentes com TNBC beneficiem do olaparib, particularmente com tumores possuindo mutações somáticas em *BRCA1/2* ou metilação do promotor destes genes.

Por outro lado, os inibidores da fosfoinositídeo 3-quinase (PI3K) (iPI3K) são fármacos que atuam ao nível da via do PI3K/Akt/mTOR, que é uma via de sinalização frequentemente mutada em CM, incluindo TNBC. Enquanto que o PI3K promove a ativação da quinase Akt, a fosfatase homóloga à tensina (PTEN) regula negativamente este processo, estando associada a funções de supressão tumoral. Recentemente, foi aprovado um iPI3K, o alpelisib, para tratamento de doentes com CM RE^+, RP^+, $HER2^-$ metastático ou avançado com mutações no gene *PIK3CA* e após progressão sob TH. Este inibidor apresenta especificidade para a isoforma α do PI3K, cuja subunidade catalítica é codificada pelo gene *PIK3CA*, e atua através de um mecanismo pró-apoptótico. É possível que o alpelisib tenha utilidade no tratamento de doentes com TNBC com mutações ao nível do *PIK3CA*.

Curiosamente, foi previamente descrita uma sinergia entre iPARP e iPI3K em modelos de TNBC, o que levou a um ensaio clínico que pretendeu testar a combinação de olaparib com os iPI3K buparlisib ou alpelisib em doentes com TNBC recorrente ou cancro ovárico seroso de alto grau. Este ensaio demonstrou existir sinergia entre olaparib e alpelisib em cancro ovárico HRR-competente. O mesmo poderá ser verdade para TNBC *BRCA*-competente, e talvez especialmente em doentes com mutações no *PIK3CA*.

O ligando do recetor ativador do fator nuclear kappa B (RANKL) é uma proteína homotrimérica pertencente à superfamília dos fatores de necrose tumoral (TNF, do inglês *tumour necrosis factors*) e que pode existir na forma membranar ou solúvel. O RANKL liga-se ao recetor RANK, um recetor homotrimérico transmembranar que requer proteínas adaptadoras para ser funcional. Destas, destaca-se o fator associado a TNF 6 (TRAF6), que tem um envolvimento crucial na ativação de vias a jusante de

RANKL/RANK, nomeadamente as vias do PI3K/Akt/mTOR, das proteínas quinases ativadas por mitogénios (MAPKs), e da Src/fosfolipase gamma (PLCγ). Esta cascata de transdução de sinal resulta na promoção da sobrevivência, diferenciação e proliferação celulares devido à translocação nuclear de ativadores transcricionais, nomeadamente o NF-κB. A via de sinalização RANKL/RANK é negativamente regulada pela osteoprotegerina (OPG), um recetor "engodo" que é capaz de se ligar ao RANKL com uma afinidade largamente superior à que o RANK apresenta.

A via do RANKL/RANK está envolvida em diversos processos fisiológicos, sendo de ressalvar a importância crucial na manutenção da homeostase óssea. Uma outra função crítica desta via ocorre ao nível do tecido mamário, nomeadamente promovendo a morfogénese da glândula mamária durante a gravidez. A progesterona possui um efeito mitogénico nas células epiteliais mamárias, ligando-se ao RP de células epiteliais luminais e promovendo um aumento da expressão de RANKL. Esta citocina vai interagir com células $RANK^+$, tanto basais como luminais, e promover fenómenos de proliferação e sobrevivência. De destaque, há duas cascatas de sinalização envolvidas neste processo, uma resultando na transcrição da ciclina D1 dependente do NF-κB, e outra resultando na redução da transcrição da p21 mediada pelo inibidor da proteína *DNA binding* 2 (Id2).

No contexto oncológico, já foi observada expressão de RANK e RANKL em diversos tumores humanos e, inclusive, foram propostas associações preditivas de mau prognóstico. O envolvimento da via RANKL/RANK neste contexto foi já descrito ao nível da promoção da transição epitélio-mesênquima (TEM), migração celular, neovascularização tumoral, estabelecimento de metástases à distância, imunossupressão, entre outros. Um dos cancros em que foi estabelecida uma relação entre esta via e a carcinogénese é o CM – tendo sido destacado o envolvimento da progesterona e derivativos sintéticos. Para além disso, foi ainda identificado o papel crucial do eixo progesterona/RANKL na tumorigénese de CM com mutações em *BRCA1*, que são maioritariamente TNBC. Ainda no contexto do CM, a via RANKL/RANK está por detrás do ciclo vicioso que se observa frequentemente nas metástases ósseas, em que ocorre uma promoção descontrolada da reabsorção óssea, criando no osso um nicho favorável ao estabelecimento das células tumorais.

A via RANKL/RANK é, deste modo, um alvo terapêutico emergente tanto ao nível do TNBC como do CM no geral. Entre 2010 e 2011, foi aprovado para utilização clínica um anticorpo monoclonal anti--RANKL totalmente humanizado, denosumab, sendo atualmente utilizado em doentes com CM sob terapia de ablação hormonal e em contexto de doença metastática óssea. Tendo sido previamente observado um efeito do bloqueio do RANKL *in vitro* na prevenção da tumorigénese de CM mutado em *BRCA1*, é possível que este bloqueio se possa demonstrar vantajoso em TNBC quando integrado em abordagens terapêuticas combinatórias.

Neste projeto, colocámos a hipótese de que a inibição farmacológica da via RANKL/RANK, através do bloqueio do RANKL, poderia apresentar sinergia com iPARP, como o olaparib, e/ou iPI3K, como o alpelisib, no contexto do TNBC.

Considerando que o TNBC pode expressar RANK e RANKL, e que uma maior expressão destas proteínas poderá estar associada a uma maior suscetibilidade ao bloqueio por RANKL, começámos por avaliar a expressão de ambas num painel de linhas celulares TNBC (BT-20, HCC1937, MDA-MB-231 e MDA-MB-468).

De seguida, de modo a justificar uma abordagem de inibição da via RANKL/RANK, foi confirmada a ativação da via por RANKL exógeno, nomeadamente a fosforilação de proteínas a jusante da via como p-65, Erk e Akt. Para realizar o bloqueio do RANKL, foi selecionada a proteína recombinante OPG-Fc, que confirmámos ser capaz de inibir a ativação da via pelo RANKL.

Estando verificada a possibilidade da abordagem inibitória, foram primeiramente realizados ensaios de viabilidade celular após 72 horas de exposição a olaparib, alpelisib e OPG-Fc, em regime individual. As diferentes linhas celulares mostraram-se, na sua maioria, resistentes ao olaparib, mas sensíveis ao tratamento com alpelisib, embora a diferentes níveis. A OPG-Fc não mostrou afetar, *per se*, a viabilidade celular. Finalmente, de modo a verificar o efeito do bloqueio do RANKL quando em combinação com olaparib e/ou alpelisib, foram realizados ensaios de viabilidade envolvendo combinações duplas de olaparib e OPG-Fc ou alpelisib e OPG-Fc; e combinações triplas de olaparib, alpelisib e OPG-Fc. Nenhuma das referidas combinações se mostrou significativamente vantajosa em termos de redução da viabilidade celular quando comparados os regimes com e sem OPG-Fc. Foi confirmado por análise de expressão proteica que o olaparib e o alpelisib estavam, de facto a afetar os respetivos alvos moleculares, confirmando-se assim que a adição de OPG-Fc não foi eficaz no contexto das células TNBC avaliadas.

Apesar dos resultados obtidos, a inibição da via do RANKL/RANK continua a ser um alvo terapêutico importante no âmbito do TNBC, existindo evidências pré-clínicas de sinergia do bloqueio do RANKL com inibidores de *checkpoints* do sistema imunitário.

Palavras-chave: cancro da mama triplo negativo; via RANKL/RANK; olaparib; alpelisib; OPG-Fc.

ABSTRACT

Triple-negative breast cancer (TNBC) accounts for around 10% to 15% of breast cancer (BC) cases and is typically associated with bad clinical outcomes. Chemotherapy is the current standard of care for this complex disease. However, high likelihood of relapse is observed due to acquired chemoresistance. Moreover, TNBC also strikingly lacks effective targeted therapies. These aspects highlight the critical importance of research on new and innovative approaches to treat these patients. Combination therapies are quite pertinent for drug research on clinically relevant alternatives. Namely, they may allow drug repurposing for faster yet effective translation into the clinical practice and also permit the administration of lower drug dosages, thus reducing side effects.

The receptor activator of nuclear factor kappa B ligand (RANKL)/RANK pathway became an emerging target in this context after being linked to TNBC tumorigenesis. This pathway is involved in cell proliferation and survival mainly through downstream activation of the NF-κB, phosphoinositide 3--kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways. Furthermore, dual RANKL and RANK expression was associated with worse prognosis amongst TNBC patients when compared to TNBC presenting RANK single expression. Here, we describe the impact of a combination regimen including RANKL blockade with osteoprotegerin (OPG)-Fc and poly (adenosine diphosphate-ribose) polymerase (PARP) inhibition with olaparib and/or PI3K inhibition with alpelisib in TNBC cell lines BT-20, HCC1937, MDA-MB-231 and MDA-MB--468. The cell line panel was initially characterised according to RANK and RANKL expression. Following exogenous RANKL stimulus, we verified pathway activation, which was successfully abrogated upon OPG-Fc treatment. We report no statistically significant impact on cell viability associated with combination therapies including RANKL blockade, PARP inhibition and/or PI3K inhibition *in vitro* in the TNBC cell lines.

Nonetheless, despite the lack of synergy observed in the selected combinations, RANK signalling remains an important target in TNBC patients, with reports of pre-clinical success with other drug types, namely immune checkpoint inhibitors.

Keywords: triple-negative breast cancer; RANKL/RANK pathway; olaparib; alpelisib; OPG-Fc.

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ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
ALP	Alpelisib
APS	Ammonium persulfate
ATR	Ataxia Telangiectasia and Rad3-related
BC	Breast cancer
BER	Base excision repair
BM	Bone metastasis
BRCA 1/2	Breast cancer 1/2
BSA	Bovine serum albumin
CCLE	Cancer Cell Line Encyclopaedia
cDNA	Complementary deoxyribonucleic acid
Chk1	Checkpoint kinase 1
DC	Dendritic cell
DFS	Disease-free survival
DMBA	Dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinase
ETS1	E26 transformation-specific 1
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FDA	United States Food and Drug Administration
GAG	Glycosaminoglycans
HER2	Human epidermal growth factor 2
HR	Hormone receptors
HRP	Horseradish peroxidase
HRR	Homologous recombination repair
HRT	Hormone replacement therapy
HT	Hormone therapy
Id2	Inhibitor of DNA binding protein 2
IGF-1	Insulin-like growth factor 1
IKK-α	Inhibitor-kappa B kinase alpha
IL	Interleukin
IκBα	Inhibitor of kappa B alpha
JNK	c-Jun N-terminal kinase
LEC	Luminal epithelial cell
LGR4	Leucine-rich repeat-containing G-protein-coupled receptor 4
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MaSC	Mammary adult stem cell

MEC	Myoepithelial cell
MEM	Minimum Essential Medium Eagle
MITF	Melanocyte inducing transcription factor
MMP	Matrix metalloproteinase
MPA	Medroxyprogesterone acetate
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NAD^+	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa B
NHEJ	Non-homologous end joining
NTC	No template control
ODF	Osteoclastic differentiation factor
OLA	Olaparib
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
OS	Overall survival
PARP	Poly (adenosine diphosphate-ribose) polymerase
PARPi	Poly (adenosine diphosphate-ribose) polymerase inhibitor
PARylation	Poly adenosine diphosphate ribosylation
PBS	Phosphate buffered saline
pCR	Pathologic complete response
PI3K	Phosphoinositide 3-kinase
PI3Ki	Phosphoinositide 3-kinase inhibitor
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKB	Protein kinase B
PLCγ	Phospholipase gamma
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
RFS	Relapse-free survival
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSB	Single-strand break
TACE	Tumour necrosis factor alpha converting enzyme
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNBC	Triple-negative breast cancer
TNF	Tumour necrosis factor
TRAF	TNF-receptor associated factors
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRANCE	Tumour necrosis factor-related activation-induced cytokine
TRANCE-R	Tumour necrosis factor-related activation-induced cytokine receptor
vWF	von Willebrand factor

1. STATE OF THE ART

Breast cancer (BC) is one of the cancer types with highest worldwide mortality rate, second only to lung cancer. It is the most common form of cancer affecting women[1] and one in every eight women is predicted to develop BC during their lifetime[2]. BC is a multifactorial disease, and its risk factors include genetic predisposition, age over 40 years, early menarche, late menopause, Caucasian race, late childbearing and some hormonal contraception and combined hormone replacement therapy (HRT) regimens[3–5]. Less than 25% of BC cases are associated with familial susceptibility, of which around 20% are due to mutations in the BC susceptibility genes *BRCA1* and *BRCA2*[6]. Female *BRCA1* and *BRCA2* mutation carriers have a risk of developing BC by the age of 80 of 72% and 69%, respectively[7].

BC is genetically and clinically heterogeneous[8], an aspect which led to its subgrouping to attempt a more specific treatment of patients. The classical immunohistochemical categorisation focuses on the expression of hormone receptors (HR), namely progesterone receptor (PR) and oestrogen receptor (ER), and human epidermal growth factor 2 (HER2, also referred to as ErbB2) amplification[9]. Based on this, BC can be divided into three major subtypes: luminal (ER^+ and/or PR^+), which can be further discriminated as luminal A ($HER2^-$, Ki-67^{low}) or luminal B ($HER2^-$, Ki-67^{high}; or $HER2^+$); HER2--overexpressing (ER^-, PR^-, $HER2^+$); and triple-negative BC (TNBC; ER^-, PR^-, $HER2^-$)[10–12]. This subtyping is of clinical relevance because it determines the possibility to use hormone therapy (HT) or HER2-targeted therapy.

1.1. Triple-negative breast cancer

TNBC is defined by lack of expression of both ER and PR and lack of HER2 amplification. Around 10% to 15% of all BC are classified as triple-negative[13]. TNBC presents an aggressive clinical course and has a worse prognosis with reported higher likelihood of distant recurrence and death within 5 years of diagnosis compared with the other subtypes[14–16]. It is associated with higher rates of brain, lung and distant nodal metastases, with bone and liver metastases less common than in non-TNBC[17]. The prevalence of TNBC is high amongst younger women, especially of African American descent[18], and around 10% to 15% of TNBC patients carry germline mutations in *BRCA1/2*[19]. Furthermore, around 70% of all *BRCA1*-mutated BC are TNBC, contrary to *BRCA2*-mutated BC which are usually ER^+ [20,21].

Chemotherapy is the current standard of care for both metastatic and non-metastatic TNBC[22–24]. TNBC is associated with higher chemosensitivity compared to non-TNBC, which is reflected in the higher pathologic complete response (pCR) rates[25,26]. However, in patients who did not achieve pCR, it is associated with a poor prognosis because of the high likelihood of early relapse due to acquired resistance to chemotherapeutic drugs[26,27]. This contrast is termed “triple-negative paradox”.

For this reason, the lack of targeted therapies is a significant constraint to the effective treatment of TNBC patients. An exception are TNBC patients with germline mutations in *BRCA1/2*, tumour suppressor genes which encode proteins with crucial functions in homologous recombination repair (HRR), for whom a targeted approach, poly-(ADP-ribose) polymerase (PARP) inhibition, is available after chemotherapy in the adjuvant and/or metastatic setting[22]. Moreover, because TNBC is also very heterogeneous, it is more likely that future therapeutic approaches will focus on a specific molecular subtype rather than the whole group of tumours. Several studies have already proposed different molecular classifications for TNBC but they have had little impact in the clinical setting so far[28–32].

1.1.1. PARP inhibitors (PARPi) in the treatment of TNBC

PARP-1 and PARP-2 are enzymes involved in base excision repair (BER), a DNA damage repair mechanism for DNA single-strand breaks (SSB) predominantly during S-phase, when DNA is exposed for replication[33]. In a process termed poly-ADP-ribosylation (PARylation), PARP-1 and PARP-2 act as catalysts of the addition of adenosine diphosphate (ADP) chains to target proteins, with nicotinamide adenine dinucleotide (NAD^+) as a substrate. PARylation of histones relaxes the chromatin, allowing for the recruitment of DNA repair proteins to sites of damage[34]. PARPi are small molecules that bind reversibly to the NAD^+ site of PARP-1 and PARP-2 and prevent PARylation, thus also preventing BER--mediated SSB repair. Moreover, there are evidences that PARPi compromise PARP-1 strand ligation, resulting in continuous turnover of PARP-1 at damage sites and increased sister-chromatid exchange[35].

During the S/G2-phase, HRR predominates over non-homologous end joining (NHEJ) regarding DNA double-strand break (DSB) repair because of the presence of a sister chromatid. HRR is regarded as relatively error-proof, contrary to the more error-prone NHEJ. However, some cancers may present impaired HRR, including BC with mutations on *BRCA1* and/or *BRCA2*[36]. As per the "two-hit" hypothesis formulated by Alfred Knudson[37], cancer cells of *BRCA* mutation carriers tend to present loss of the healthy *BRCA1/2* allele through somatic mutation or epigenetic silencing, in a process named loss of heterozygosity (LOH), which represents the second "hit" for tumour suppressor inactivation and impairs HRR of DSB[38,39]. PARPi are effective in *BRCA1/2*$^{-/-}$ cancer cells via synthetic lethality: inhibition of PARP leads to the accumulation of SSB, whilst preventing their repair and therefore resulting in the formation of DSB; simultaneously, the intrinsic deficiency in HRR presented by cancer cells leads to increased apoptosis due to incorrect DNA repair. A very significant advantage of PARPi--related synthetic lethality resides in its specificity – because non-cancerous cells are heterozygous for *BRCA1/2* mutations, the toxicity is focused on cancer cells[40].

In the context of BC, the PARPi olaparib (Lynparza®, AstraZeneca Pharmaceuticals LP) was approved by the US Food & Drug Administration (FDA, in 2018) and the European Medicines Agency (EMA, in 2019) as monotherapy for the treatment of (1) patients with germline *BRCA1/2* mutations presenting $HER2^-$ metastatic or locally advanced BC and previously treated with a taxane and an anthracycline (two chemotherapeutic agents) unless not suitable for these treatments; and also (2) patients with HR^+ BC showing tumour progression on or after endocrine therapy, or otherwise considered unsuitable for said approach. Olaparib had previously been approved, in 2014, for patients with ovarian, fallopian and peritoneal cancer, namely those with *BRCA* mutations, and more recently, in 2020, for patients with metastatic pancreatic cancer presenting germline *BRCA* mutations[41–43].

PARP inhibition is the only targeted approach currently approved for use in a subset of TNBC patients with germline *BRCA* mutations. However, other TNBC patients might also benefit from olaparib, namely those presenting somatic *BRCA* mutations[44] or *BRCA* wild type with gene inactivation through promoter methylation[45,46].

1.1.2. PI3K inhibitors (PI3Ki) in the treatment of TNBC

The phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is an important regulator of glucose metabolism and cell growth physiologically triggered upstream through stimulation of receptor tyrosine kinases by growth factors such as insulin[47]. It is the most frequently activated signalling pathway in BC and is mutated in around 40% of TNBC patients[48].

The *PIK3CA* gene encodes for the p110α subunit of PI3Kα and was reported as the second most common gene mutation amongst TNBC, presenting activation in around 20% to 25% of cases[49–51]. There are many isoforms of PI3K, which have been divided into three classes (I, II and III) according to structural characteristics and substrate preferences. However, the class I PI3K isoforms, composed of a p85 regulatory subunit and a p110 catalytic subunit[52,53], are the most commonly altered in cancer. In cancer, these protein complexes may be mutated so as to activate the signalling pathway, and this can occur either by inactivation of p85 or by overactivation of p110[54], which is the case of *PIK3CA* mutations.

Phosphatase and tensin homologue (*PTEN*) is a tumour suppressor gene encoding for a lipid phosphatase that regulates the PI3K/Akt/mTOR pathway through dephosphorylation of phosphoinositide-3,4,5-trisphosphate (PIP3)[55], opposing PI3K activation of protein serine/threonine kinase Akt (also called protein kinase B, PKB)[56,57]. Cancer cells benefit from the inactivation of *PTEN*, which can either occur through mutation[58] or promoter hypermethylation[59].

Alpelisib (Piqray®, Novartis Pharmaceuticals Corporation) is a PI3Ki with specificity towards p110α[60], and because it is an isoform-specific inhibitor it presents less off-target adverse effects when compared with pan-PI3K inhibitors[54]. It induces apoptosis through a mechanism involving Akt-dependent induction of Bim, a pro-apoptotic protein, and degradation of Mcl-1, an apoptosis inhibitor[61]. Alpelisib was approved by the FDA in 2019 and, very recently, by the EMA (in 2020), in combination with the ER antagonist fulvestrant for the treatment of men and postmenopausal women with HR^+, $HER2^-$ metastatic or locally advanced BC presenting *PIK3CA* mutations, following progression after endocrine monotherapy[62,63]. Despite clinical data showing no benefit in the addition of a pan-PI3Ki to a chemotherapeutic regimen for TNBC patients[64,65], PI3Ki might still have value in combination strategies, since in these studies no selection was made for *PIK3CA*-mutated TNBC.

1.1.3. PARPi and PI3Ki synergy

Interestingly, in 2012 two studies described *in vitro* and *in vivo* synergy between PI3Ki, using buparlisib (NVP-BKM120, a pan-PI3Ki), and PARPi, using olaparib, in *BRCA* competent or mutant TNBC models. PI3K inhibition was accompanied by downregulation of *BRCA1/2* and an increase in DNA damage and PARylation, reflecting an abrogation of HRR and a dependency on PARP activity, thus sensitising cells to PARP inhibition. *BRCA* downregulation appeared to be mediated by an extracellular signal-regulated kinase (ERK)-dependent activation of the transcription factor E26 transformation--specific 1 (ETS1), thereby suppressing gene transcription[66,67]. Additionally, PI3Ki impair the production of nucleotides required for DNA repair and synthesis, which could also lead to an increased DNA damage when combined with PARPi[68]. Based on these findings, the phase I clinical trial NCT01623349 focused on the combination of olaparib with PI3Ki (buparlisib or alpelisib) for the treatment of patients with recurrent TNBC or high grade serous ovarian cancer. Results from this ongoing study have provided clinical evidence of synergy between olaparib and alpelisib in HRR--proficient ovarian cancer[69]. However, *BRCA*-mutated ovarian cancer showed little improvement from the combination regimen versus olaparib monotherapy. This suggests that the combination regimen could be effective for *BRCA*-competent TNBC, especially in *PIK3CA*-mutated cases.

1.2. RANKL/RANK pathway

Receptor activator of nuclear factor kappa-B ligand (RANKL; also known as osteoprotegerin ligand (OPGL), TNF-related activation-induced cytokine (TRANCE) and osteoclastic differentiation factor

(ODF)[70–73]) is a member of the tumour necrosis factor (TNF) superfamily encoded by the *TNFSF11* gene (13q14) that binds to the signalling receptor RANK (*TNFRSF11A* gene, 18q22.1)[70,74]. RANKL is a homotrimeric type II membrane protein which can occur in three isoforms: RANKL1, the full-length structure; RANKL2, a shorter form missing part of the intra-cytoplasmic domain; and RANKL3, a soluble isoform missing the N-terminal[75]. Besides alternative splicing, membrane-bound RANKL can be shed in a soluble form through proteolysis catalysed by enzymes such as TNF-α-converting enzyme (TACE), A disintegrin and metalloproteinase (ADAM)-10, matrix metalloproteinase (MMP)-7 and MMP-14[76–78]. RANKL is highly expressed in bone tissue, lymphoid organs and the vascular system, but is also present in other tissues such as the brain, breast, intestine, kidney, liver, lung and skeletal muscle[79,80]. Its expression can be induced by factors including progesterone, prolactin, parathyroid hormone-related protein (PTHrP), vitamin D3, TNF-α and the interleukins (IL)-11 and 17[81,82].

RANK (or TRANCE receptor (TRANCE-R)), on the other hand, is mainly expressed in the bone, although *RANK* messenger RNAs (mRNAs) have been found in other tissues such as breast, liver, prostate and thymus[74,83]. It is a homotrimeric type I transmembrane protein and, similarly to other members of the TNF receptor superfamily, RANK lacks tyrosine kinase activity, requiring adapter proteins, the TNF receptor-associated factors (TRAF), to be functional[84]. RANK possesses intracellular TRAF binding sites which are able to interact with TRAF-1, 2, 3, 5 and 6[84–86]. Amongst these, TRAF6 has been shown to have a predominant role in RANK-associated signalling[87], leading to the activation of several important transduction pathways, namely PI3K/Akt/mTOR; mitogen-activated protein kinases (MAPKs), including p38, c-Jun N-terminal kinase (JNK) and ERK1/2; and Src/phospholipase gamma (PLCγ). This leads to the nuclear translocation of transcriptional activators, primarily nuclear factor kappa-B (NF-κB) but also others such as Fos/Jun and melanocyte inducing transcription factor (MITF), and results in the transcription of numerous effector genes associated with cell survival, differentiation and proliferation[86,88,89]. Several RANK alternative splicing isoforms have been described, of which RANK-c stands out due to its ability to counteract RANK-mediated NF-κB activation[90,91].

The RANKL/RANK pathway is negatively regulated by the soluble decoy receptor osteoprotegerin (OPG), encoded by the *TNFRSF11B* gene[92]. Despite its ubiquitous expression, OPG is detected predominantly in bone, immune cells and the vascular system[83,93]. OPG is able to bind RANKL with an affinity approximately 500 times superior to that of RANK, facilitating the internalization of the cytokine and thus reducing its half-life[94,95]. OPG can be induced by factors such as oestrogen, IL-4 and transforming growth factor beta (TGF-β)[96], besides being controlled by many ligands including TNF--related apoptosis-inducing ligand (TRAIL)[97], glycosaminoglycans (GAGs)[98] and von Willebrand factor (vWF)[93], all of which will influence the inhibitory effect. Moreover, in 2016, Luo *et al.* identified a new RANKL receptor, leucine-rich repeat-containing G-protein-coupled receptor 4 (LGR4), involved in a feedback loop that, like OPG, contributes to the control of RANKL activity[99].

1.2.1. Physiological functions

The RANKL/RANK axis was originally discovered to mediate T-cell and dendritic cell (DC) communication. Activated T-cells express RANKL, which interacts with RANK expressed on DCs, increasing their antigen-presenting capacity. This leads to an increase in the number of antigen-specific T-cells and enhances memory T-cell immunity[74]. Moreover, RANKL acts as a specific survival factor for DCs[72] and is essential for the development of lymph nodes[100] and thymic function[101].

However, its most prominent role is in the bone. It was discovered to be crucial for the regulation of bone formation and resorption, maintaining bone homeostasis[100]. Osteoblasts are bone-forming mononucleated cells of mesenchymal origin, which, together with osteocytes, chondrocytes and stromal cells, express RANKL. RANKL binds to its cognate receptor RANK expressed in osteoclast precursor cells, of hematopoietic origin, thereby inducing osteoclast survival and osteoclastogenesis, and subsequent osteoclast-mediated bone resorption. OPG, also produced by osteoblasts, inhibits bone resorption and, therefore, a balanced OPG:RANKL ratio contributes to bone health, avoiding osteoporosis (OPG<RANKL), commonly seen in postmenopausal women, and osteopetrosis (RANKL>OPG)[70,71,100,102].

The RANKL/RANK pathway has also been associated with other physiological processes, e.g. in the central nervous system[103] and in the breast, where RANKL was discovered to be a crucial regulator of the proliferation and differentiation of mammary epithelial cells especially during pregnancy, driving the morphogenesis of the lactating mammary gland[81]. The mammary epithelium comprises an outer basal layer containing myoepithelial cells (MECs) and adult mammary stem cells (MaSCs), and an inner luminal layer consisting of luminal epithelial cells (LECs). Whereas LECs line the ducts and alveoli and are involved in milk synthesis and secretion during lactation, MECs facilitate milk excretion due to their contractile phenotype and MaSCs are able to regenerate the entire mammary epithelial tree due to their self-renewable and multipotent potential[104,105]. Progesterone, a steroid hormone presenting highest levels during the luteal phase of the menstrual cycle and throughout pregnancy[106], is known to have mitogenic effects on the mammary epithelial cells (**Figure 1.1a**) through its binding to the PR in PR^+ LECs, upregulating RANKL expression due to mRNA stabilisation[107]. The newly synthesised RANKL acts in LECs, which are $RANK^+$, further upregulating RANKL; and in a paracrine fashion on the HR^- MECs and MaSCs, also $RANK^+$. Upon RANKL-RANK binding, two main pathways are triggered on mammary epithelial cells (**Figure 1.1b**). On one hand, it leads to the activation of inhibitor kappa-B kinase alpha (IKK-α), which induces proteasome degradation of inhibitor of kappa-B alpha (IκBα). Its dissociation from NF-κB allows the latter to migrate to the nucleus and induce transcription of cyclin D1, involved in the G1 phase of the cell cycle. On the other hand, it also promotes the nuclear translocation of inhibitor of DNA binding protein 2 (Id2), a transcriptional regulator which downregulates p21, a cell cycle inhibitor[108–110]. Additionally, the binding of RANKL to RANK expressed in the surface of basal cells upregulates RANK expression, notably contributing even more to the proliferation and expansion of MaSCs[111]. Besides progesterone, prolactin and PTHrP can also promote RANKL expression in the mammary epithelium[81].

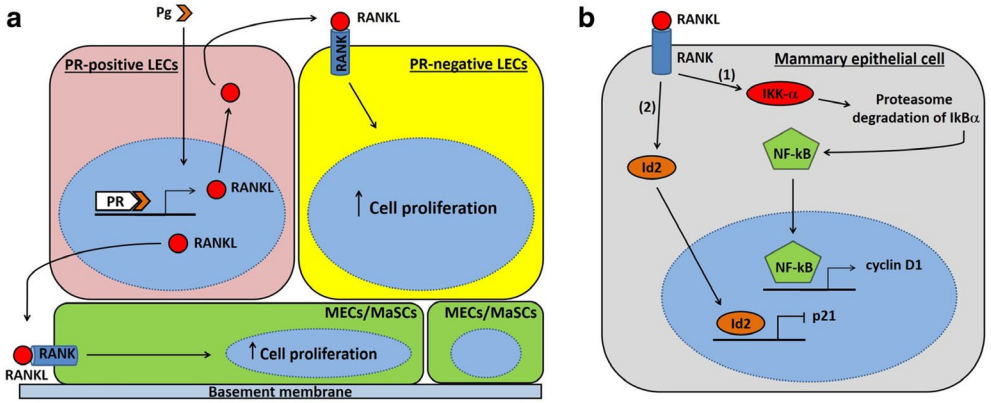

Figure 1.1 Physiological involvement of the RANKL/RANK pathway in mammary epithelial cells. (**a**) Progesterone binds to PR in PR^+ luminal epithelial cells and upregulates RANKL expression, which in turn activates RANK in $RANK^+$ luminal and basal cells, promoting proliferation and survival. (**b**) RANKL binding to RANK in mammary epithelial cells triggers two main cascades leading to NF-κB-dependent cyclin D1 transcription and Id2-mediated p21 downregulation. Adapted from Infante *et al.*, 2019[112].

1.2.2. Involvement in cancer

Numerous studies have demonstrated RANK and/or RANKL expression in different tumours[113–115], and others have even described this expression as predictive of poor prognosis[116–118]. Together with observations regarding physiological functions involving cell survival and proliferation, this hints at a potential tumorigenic involvement of the RANKL/RANK/OPG axis. Rightfully so, this pathway has been implicated in every stage of cancer development, promoting events such as epithelial-mesenchymal transition (EMT) and stemness, cell migration, neovascularization of tumours, establishment of distant metastases, and even promoting immunosuppressive responses[119–125].

The Women's Health Initiative and the Million Women Study showed an increased BC risk associated with HRT and contraceptives containing synthetic progesterone derivatives (progestins) such as medroxyprogesterone acetate (MPA)[4,5]. A 2010 study by Schramek *et al.* showed a significant induction of RANKL expression in mammary epithelial cells of female mice treated with MPA and 7,14--dimethylbenz[a]anthracene (DMBA), a carcinogen. This resulted in marked cell proliferation and eventually BC tumorigenesis. However, genetic inactivation and deletion of RANK in the mammary epithelial cells prevented the MPA-associated proliferation and delayed the onset and incidence of MPA-driven BC[126]. These results show an involvement of the RANKL/RANK pathway in progesterone and progestin-driven breast carcinogenesis, an observation further supported by Gonzalez-Suarez *et al.* in the same year[127]. In accordance, BC is one of the cancers in which RANK and RANKL expression has been documented. Moreover, RANK overexpression in normal human mammary epithelial cells was shown to induce mammary gland reconstitution, EMT, migration, stemness and anchorage--independent growth, contributing to cell transformation and metastasis[125]. A posterior study in mice found that the activation of RANK signalling led to expansion of the luminal and basal mammary compartments, resulting in the accumulation of MaSCs, luminal and bipotent progenitors. This impairment of mammary cell fate eventually resulted in hyperplasia and tumorigenesis[128].

In 2016, two studies identified a crucial role for the RANKL/RANK pathway in the tumorigenesis of *BRCA1* mutation-driven breast cancer, since *RANK* deletion and the inhibition of the pathway appeared to prevent the development of the disease[129,130]. Notably, since *BRCA1* mutations are commonly associated with TNBC[20,21], a correlation between TNBC and the RANKL/RANK pathway was established. The description of an association between RANK/RANKL dual expression in TNBC with a worse prognosis, when compared to patients with $RANK^+$, $RANKL^-$ TNBC, strengthened this observation[116]. One of the proposed mechanisms behind BC tumorigenesis involving *BRCA1* mutations and the RANKL/RANK pathway (**Figure 1.2**) is the following: (i) mutation carriers are prone to LOH, spontaneously losing the remaining wild type *BRCA1* allele; (ii) the resulting HRR-incompetent *BRCA1*-deficient cells present increased genomic instability and DNA damage; (iii) during pregnancy, the menstrual cycle or HRT, progesterone or progestins stimulate the proliferation of $RANK^+$ luminal progenitor cells via RANKL/RANK; (iv) as a result, the cells acquire genetic mutations, e.g. in the *TP53* gene, culminating in uncontrolled proliferation and development of BC[82].

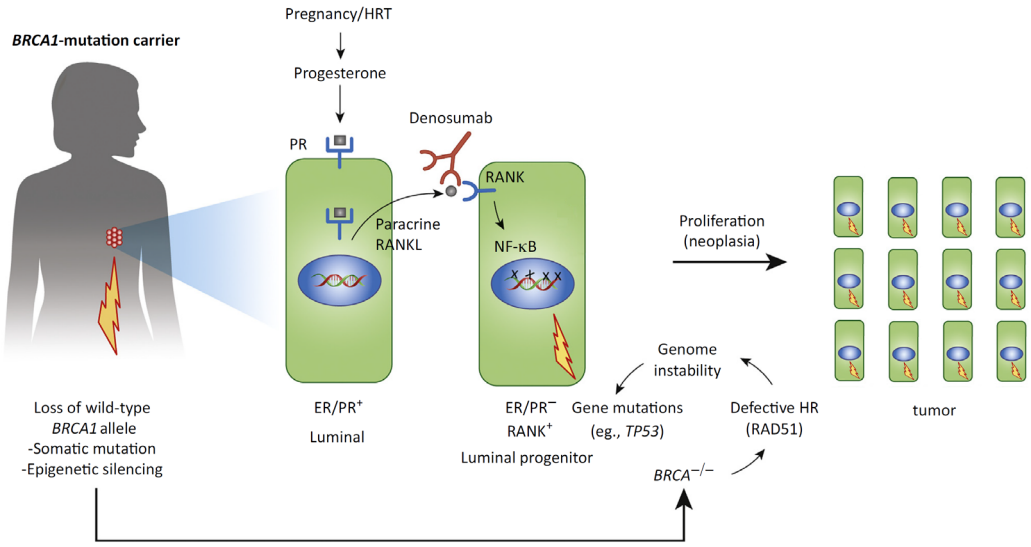

Figure 1.2 Proposed mechanism for progesterone/RANKL-mediated *BRCA1*^{mut} breast cancer carcinogenesis. Mammary epithelial cells of *BRCA1* mutation carriers may present impaired homologous recombination due to loss of the healthy *BRCA1* allele. The subsequent genomic instability increases the chances of acquiring mutations, an event which is further promoted by progesterone/progestin stimuli. This way, RANKL/RANK-mediated proliferation of *BRCA1* deficient cells may lead to the generation of driver mutations, originating breast cancer. Adapted from Rao *et al.*, 2018[131].

Besides primary cancers, the RANKL/RANK pathway is very important in the context of bone metastases (BM), with bone being the most common site of relapse in BC patients. Indeed, this pathway has been shown to promote BC-associated BMs[132]. Circulating BC cells, originating from the primary tumour, are attracted to the bone by chemokines[133]. Several bone cells, such as osteoblasts, osteocytes and stromal and lining cells, secrete RANKL, activating osteoclast-mediated bone resorption. Bone resorption releases growth factors including TGF-β and insulin-like growth factor 1 (IGF-1), which in turn stimulate cancer cell proliferation and production of several cytokines like PTHrP and IL-6. In turn, these cytokines bind to stromal cells, osteoblasts and other RANKL-producing cells to upregulate RANKL expression. This establishes a "vicious cycle" of osteolytic damage (**Figure 1.3**). Even though osteoblastic (associated with enhanced bone deposition) or mixed bone lesions may also occur, the described osteolytic metastases (associated with increased bone degradation) account for the majority of metastatic BC cases[134–137]. BMs are a good example of the "seed and soil" hypothesis, where Stephen Paget suggested that when tumour cells leave the primary tumour, they usually target organs with favourable characteristics[138].

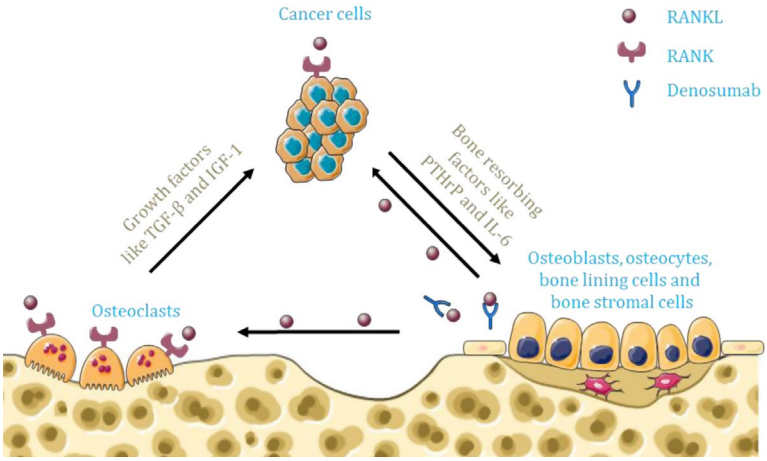

Figure 1.3 The vicious cycle of bone metastases. RANKL produced by stromal cells and osteoblasts promotes osteoclast--mediated bone resorption, releasing molecules that promote cancer cell proliferation. In turn, cancer cells secrete factors which enhance RANKL production even further, giving continuity to the cycle. Adapted from de Groot *et al.*, 2018[137].

1.2.3. Pathway blockade

The involvement of the RANKL/RANK pathway in several aspects of cancer biology suggests a possible therapeutic potential in its inhibition. Between 2010 and 2011, denosumab, a fully humanised IgG2 anti-RANKL monoclonal antibody, was approved by the FDA and the EMA for clinical use. It was proven effective in breast and prostate cancer patients receiving hormone ablation therapy, and in the prevention of skeletal-related events in multiple myeloma patients and patients with BMs from solid tumours. By blocking the binding of RANKL to RANK, denosumab is able to reduce bone resorption, mimicking the action of OPG[139–142].

Evidence suggest an anti-tumour effect for RANKL blockade either through direct inhibition of the pathway in RANK and RANKL-expressing tumours such as lung cancer[143]; by osteoclast-dependent modulation of the bone microenvironment as happens in BMs[143]; or even through the effect of the inhibition on non-cancerous cells, such as immune cells[144]. It is the case of the preclinical works that described the involvement of the RANKL/RANK pathway in *BRCA1* mutation-driven BC, in which, as previously mentioned, RANKL inhibition was able to reduce the expansion of mammary progenitors and prevent BC development[129,130]. This hints at a possible therapeutic application of RANKL blockade in TNBC chemoprevention, and possibly also in combination therapies.

2. AIMS OF THE PROJECT

As previously mentioned, since the RANKL/RANK pathway has been implicated in the tumorigenesis of TNBC, pharmacological inhibition of this pathway could be of clinical relevance beyond bone metastatic disease. We hypothesised it could synergise with PARP and/or PI3K inhibitors, which are not currently in use for all TNBC patients.

Therefore, the main objective of this project was to verify whether RANKL blockade could improve the efficacy of PARPi and PI3Ki, in combination therapy, in *in vitro* TNBC models.

Besides sensitisation of cells to other drugs and/or increasing efficacy in already sensitive cells, a combination therapy based on RANKL blockade may include some advantages that have been associated with combination regimens, including minimizing toxicity and allowing for drug repurposing, such as proposed in the current project, to allow for a faster introduction into the clinical setting[145,146].

3. MATERIALS AND METHODS

3.1. Cell culture

The human TNBC cell line panel was selected according to the mutational status of *BRCA1/2*, *PIK3CA* and *PTEN* (**Table S1**), which may influence drug response. The MDA-MB-231$^{GFP+/Luc+}$ cell line (hereby referred as MDA-MB-231) was kindly provided by Sérgio Dias (Instituto de Medicina Molecular, Lisbon, Portugal); the BT-20 cell line was purchased from ATCC (HTB-19^{TM}); and the MDA-MB-468 and HCC1937 cell lines were generously supplied by Rita Fior (Champalimaud Centre for the Unknown, Lisbon, Portugal). All cell lines were tested for *Mycoplasma* contamination.

The cell lines were cultured in media supplemented with 10% (v/v) Foetal Bovine Serum (FBS, Gibco) and 1% (v/v) Penicillin/Streptomycin (Pen/Strep: 10.000 U/mL Penicillin, 10.000 µg/mL Streptomycin; Gibco). Dulbecco's Modified Eagle Medium (DMEM) 1X containing D-Glucose, L-Glucose and Pyruvate (Gibco) was used for the MDA-MB-231 and MDA-MB-468 cell lines; Minimum Essential Medium Eagle (MEM) 1X containing Earle's Salts and L-Glutamine (Gibco) for the BT-20 cell line; and Roswell Park Memorial Institute Medium (RPMI) 1640 1X (Gibco) containing L-Glutamine for the HCC1937 cell line. Cells were maintained in T25 or T75 cell culture flasks (Thermo Scientific) at 37ºC with 5% CO_2 and humified atmosphere. Cells were used at low passages and media was changed every 2 to 3 days.

3.2. Viability assays

To assess viability, cells were seeded in 96-well plates (Costar or TPP) with a density of 10^4 cells/well and treated with different concentrations of olaparib (#10-2154, Focus Biomolecules), alpelisib (#ENZ--CHM214, Enzo Life Sciences) and OPG-Fc (Amgen, Inc.). Olaparib was solubilised in absolute ethanol; alpelisib in DMSO; and OPG-Fc in PBS. After incubation for 72h under standard conditions, cell viability was assessed using the Alamar Blue viability reagent (Invitrogen). 1:10 (v/v) reagent was added to the media of each well and, after a 2h incubation at 37ºC, fluorescence (excitation 560 nm / emission 590 nm) was read in a microplate reader (Infinite M200, TECAN). Every experiment was done in triplicate or more, with six replicates per experiment.

3.3. Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

For *RANK* and *RANKL* mRNA expression analysis, total RNA was extracted with the NZY Total RNA Isolation kit (Nzytech), according to manufacturer's instructions, and quantified using a spectrophotometer (NanoDrop 1000, Thermo Fisher). Total RNA was treated with RQ1 RNase-free DNase (Promega) for 30 min at 37ºC, in accordance with manufacturer instructions. 480 ng of DNase I-treated RNA were used for complementary DNA (cDNA) synthesis, using the NZY M-MuLV First--Strand cDNA Synthesis kit (Nzytech), Oligo(dT)$_{18}$ primer mix (Nzytech) and a thermal cycler (C1000, Bio-Rad). The resulting cDNAs were amplified by real-time PCR with TaqMan Gene Expression Master Mix (Nzytech) and specific primers including: *TNFRSF11A* (#Hs00921372_m1, Applied Biosciences), *TNFSF11* (#Hs00243522_m1, Applied Biosciences) and *GAPDH* (#PPH00150F, SA Biosciences). No template control (NTC) and RNA template controls were included. Every cDNA was tested in triplicate in 384-well PCR plates (Applied Biosystems) and amplification was performed in a Real-Time PCR System (ViiA 7, Applied Biosystems). Gene expression was averaged and normalised using the selected housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and relative mRNA expression is presented as $2^{-\Delta Ct}$.

3.4. Flow cytometry

To evaluate RANK expression by flow cytometry, cells were resuspended and centrifuged twice for 5 min at 200 x g and 4ºC, with a cold PBS wash in between. The pellet was resuspended in fluorescence--activated cell sorting (FACS) buffer (2% FBS, 1X PBS) and divided into three tubes: (A) one for the desired staining; (B) one for the secondary antibody only; and (C) one for the unstained control. In tube A, cells were incubated in the dark for 30 min at 4ºC with 5 µL mouse monoclonal anti-RANK antibody (#M331, Amgen Inc.). After washing again with cold PBS, tubes A and B were incubated, also in the dark, for 30 min with Cy5-conjugated AffiniPure goat anti-mouse IgG (#115-175-205, Dianova) diluted 1:100 (v/v) in FACS buffer. Tubes A and B were then washed in FACS buffer with a similar centrifugation as before and all pellets, including from tube C, were resuspended in FACS buffer. Data acquisition was performed in a flow cytometer (LSRFortessa, BD Biosciences) and further analysis was made with the FlowJo vX.0.7 software.

3.5. Western blot

To analyse RANKL/RANK pathway activation with or without OPG-Fc blockade, $2x10^5$ cells/well were seeded in 6-well plates (Costar) for 24h and serum-starved in 0.1% FBS, 1% Pen/Strep media for another 24h. The cells were then stimulated for 20 and 60 min with media containing 1 µg/mL human RANKL (hRANKL) (Amgen Inc.) and 60 min with neutralisation media. Two neutralisation media were prepared and incubated for 1h at 37ºC: 1 µg/mL hRANKL with 2.5 µg/mL OPG-Fc; and 1 µg/mL hRANKL with 2.5 µg/mL mouse monoclonal anti-hRANKL (#MAB626, R&D Systems; hereby referred as MAB626).

To assess the molecular effect of PARP inhibition using olaparib and PI3K inhibition using alpelisib, $4x10^5$ cells/well were plated in 6-well plates and treated for 72h with 10 µM olaparib, 20 µM alpelisib, 100 ng/mL OPG-Fc and all possible combinations.

Proteins were extracted with RIPA buffer (Sigma) with 1:100 (v/v) protease inhibitor cocktail (Cell Signaling) and 1:100 (v/v) phosphatase inhibitor cocktail (Sigma) (herein referred as RIPA buffer), according to manufacturer's instructions. Protein was quantified with the Pierce BCA Protein Assay kit (Thermo Scientific), in accordance with manufacturer's instructions. Protein concentration was calculated using an albumin standard curve and protein extracts were stored at -20ºC until usage.

2X or 4X sample buffer with 5% β-mercaptoethanol was added to the extracts before a 10-minute incubation at 95ºC. Proteins were resolved on 10% or 12% SDS-PAGE gels and the NZYColour Protein Marker II (Nzytech) was used. Proteins were transferred to nitrocellulose membranes (Invitrogen) using a dry blotting system (iBlot 2, Invitrogen) and visualised with Ponceau S (Sigma) staining after transfer. Membranes were blocked for 1h in PBST (0.05% Tween 20, 1X PBS) 5% (m/v) Albumin Bovine Fraction V (BSA) (Nzytech), hereby designated as BSA; or in PBST 5% (m/v) non-fat dry milk (Molico), hereby designated as milk. Incubation with primary antibodies was overnight at 4ºC and with secondary antibodies was 2h at room temperature. The following primary antibodies were used: mouse monoclonal anti β-Actin antibody (1:25000 in milk; #ab6276; Abcam), rabbit monoclonal anti-NF-kB p65 (D14E12) (1:1000 in BSA; #8242, Cell Signaling), rabbit monoclonal anti-Phospho-NF-kB p65 (Ser536) (93H1) (1:1000 in BSA; #3033, Cell Signaling), mouse monoclonal anti-IkBα (L35A5) (1:1000 in BSA; #4814, Cell Signaling), rabbit monoclonal p44/42 MAPK (Erk1/2) (137F5) (1:1000 in BSA; #4695, Cell Signaling), rabbit polyclonal anti-Phospho-ERK1/2 (Thr 202/Tyr 204) (1:1000 in BSA; #sc-16982, Santa Cruz Biotechnology), rabbit monoclonal anti-Akt (pan) (11E7) (1:1000 in BSA; #4685, Cell Signaling), rabbit monoclonal anti-Phospho-Akt (Ser473) (D9E) (1:1000 in BSA; #4060,

Cell Signaling), rabbit polyclonal anti-hsRANKL (1:5000 in BSA; #500-P133, PeproTech), rabbit monoclonal anti-Phospho-Chk1 (Ser345) (133D3) (1:500 in BSA; #2348, Cell Signaling), mouse monoclonal anti-S6 (H-4) (1:500 in BSA; sc-74576, Santa Cruz Biotechnology), rabbit polyclonal anti--Phospho-S6 (Ser240/244) (1:1000 in BSA; #2215, Cell Signaling). Also, the following horseradish peroxidase (HRP)-conjugated specific secondary antibodies were used: anti-mouse-HRP IgG (1:5000 in milk; #7076, Cell Signaling), and anti-rabbit-HRP IgG (1:5000 in milk; #7074, Cell Signaling).

Target proteins were detected using the Novex ECL HRP Chemiluminescent Substrate Reagent kit (Invitrogen) in a blot and gel imager (Amersham Imager 680, GE Healthcare). Some membranes were stripped with membrane stripping solution (Nzytech) and reused. Band intensity was calculated using the ImageJ 1.48v software and normalised for β-actin and, when applicable, to total protein.

3.6. Statistical analysis

GraphPad Prism software version 8.4.3 was used for data analysis and construction of graphics. For the cell viability assays, one-way ANOVA was performed. Values are presented as mean ± SEM and $p<0.05$ was considered for statistical significance.

4. RESULTS AND DISCUSSION

4.1. RANK and RANKL expression in TNBC cell lines

TNBC has been shown to express RANK and RANKL[116]. We started by quantifying endogenous RANK expression across a TNBC cell line panel, by RT-qPCR and flow cytometry (**Figure 4.1**); and endogenous RANKL expression by RT-qPCR and western blot (**Figure 4.2**).

In accordance with data available in the Broad Institute Cancer Cell Line Encyclopedia (CCLE) (**Table S2**), MDA-MB-468 and MDA-MB-231 cell lines presented the highest *RANK* mRNA expression amongst the panel, with HCC1937 and BT-20 cell lines presenting lower expression (**Figure 4.1A**). RANK protein expression was further confirmed through surface protein staining (**Figure 4.1B**).

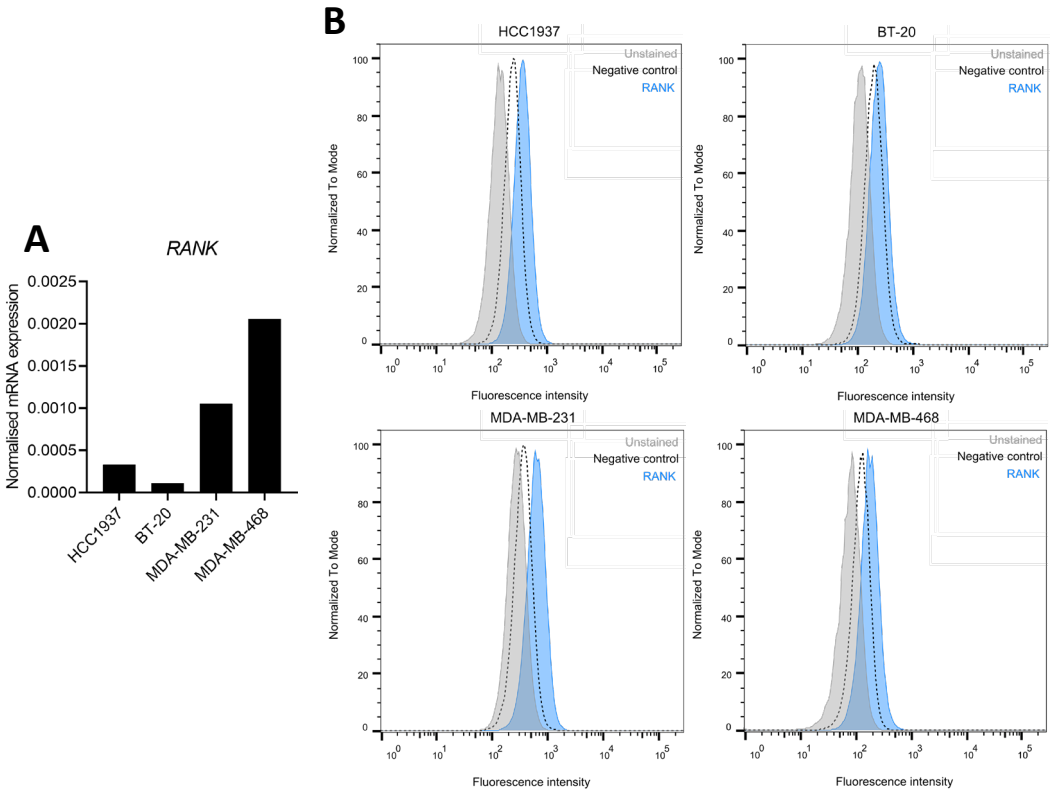

Figure 4.1 Analysis of *RANK* expression in TNBC cell lines. (**A**) RT-qPCR of *RANK* in HCC1937, BT-20, MDA-MB-231 and MDA-MB-468 cells. Relative mRNA expression was calculated as $2^{-\Delta Ct}$ using the housekeeping gene *GAPDH* for normalisation. (**B**) Flow cytometry of RANK in the TNBC panel.

Regarding RANKL expression, HCC1937 cells presented the highest *RANKL* mRNA levels (**Figure 4.2A**), again in agreement with data from the CCLE (**Table S2**). However, we did not observe significant differences in protein expression assessed by western blot, even in comparison to MCF-7 luminal BC cells, known to express RANKL (**Figure 4.2B**). RANKL is a homotrimeric protein which can also be shed in a soluble form. The western blot for RANKL presents multiple bands that correspond to multimeric forms of higher molecular weight, which could include dimers and trimers, whereas bands around 35 kDa are possibly monomeric forms of RANKL. Therefore, bands at lower molecular weights should correspond to its soluble form, considering soluble RANKL possesses a shorter structure[75].

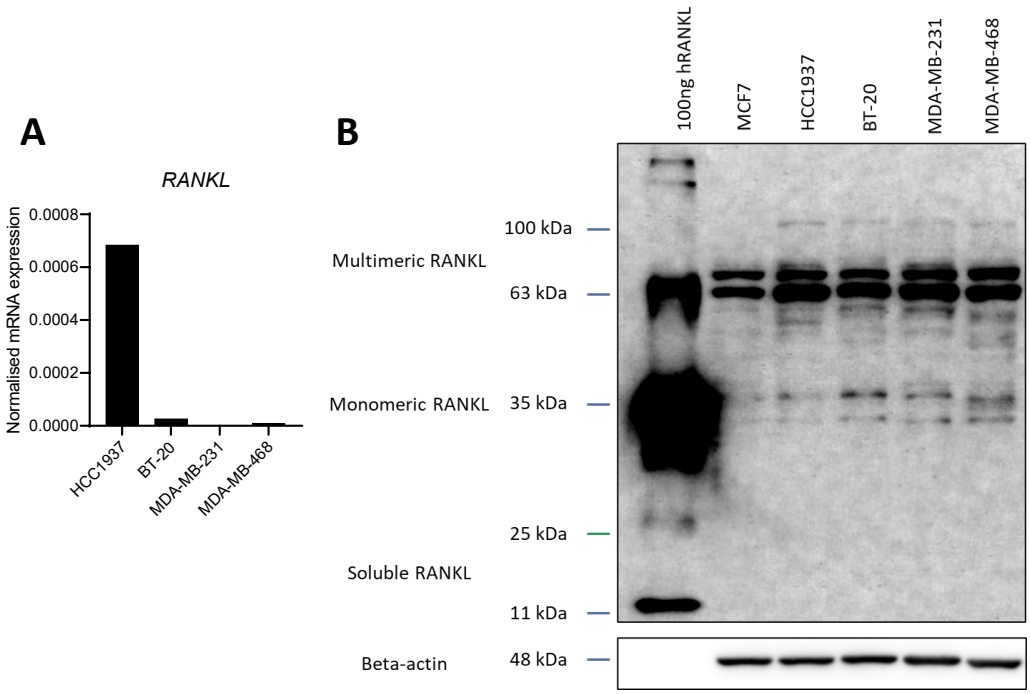

Figure 4.2 Analysis of RANKL expression in TNBC cell lines. (**A**) RT-qPCR of *RANKL* in HCC1937, BT-20, MDA-MB--231 and MDA-MB-468 cell lines. Relative mRNA expression was calculated as $2^{-\Delta Ct}$ using the housekeeping gene *GAPDH* for normalisation. (**B**) Western blot of RANKL in the selected TNBC panel with β-actin as loading control; 100 ng of hRANKL were used as a positive control; and protein extract from MCF-7 cells was used as a positive luminal BC control.

TNBC have been reported to express higher levels of surface RANK and *RANK* mRNA compared to non-TNBC, and dual expression of RANK and RANKL was associated with worse overall survival (OS) and relapse-free survival (RFS; also called disease-free survival, DFS) compared to RANK single expression[16,116]. Cell lines with highest expression of these two molecules might be more susceptible to inactivation of the RANKL/RANK pathway, namely through RANKL blockade.

4.2. RANKL/RANK pathway activation by exogenous RANKL

To assess the possibility of an inhibitory pharmacological approach via RANKL blockade, activation of the RANKL/RANK pathway by exogenous RANKL was confirmed by western blot in serum-starved cell lines of the selected TNBC panel (**Figure 4.3**).

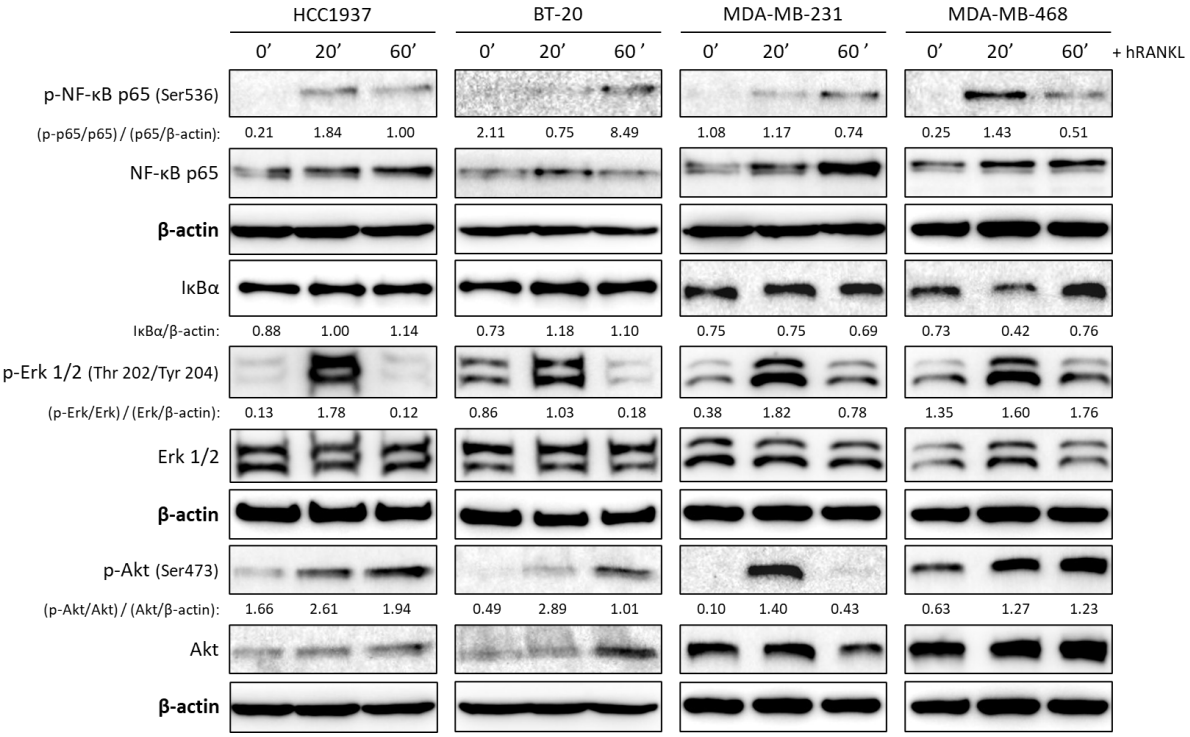

Figure 4.3 The RANKL/RANK pathway is activated by exogenous RANKL. Phosphorylation of downstream targets of the RANKL/RANK pathway and degradation of IkBα were assessed by western blot upon stimulation with 1 µg/mL hRANKL for 20 or 60 minutes. β-actin was included as loading control. Band intensity was quantified with the ImageJ software and ratios to loading control or total protein were calculated.

Following RANK activation by RANKL, IKK-α is phosphorylated and induces the degradation of IκBα by the proteasome, ultimately allowing for NF-κB translocation into the nucleus, a process requiring NF-κB subunit p65 phosphorylation[108]. Besides signalling through the NF-κB pathway, RANK also activates the PI3K/Akt/mTOR pathway via TRAF6 and c-Src, leading to the phosphorylation and activation of Akt[89]. Additionally, the MAPK/Erk pathway is also activated downstream of RANK[147]. Accordingly, we evaluated the activation of RANK pathway by exogenous RANKL by assessing the levels of total IκBα, phospho (p)-NF-κB p65 subunit, phospho-Akt and phospho-Erk. Following RANKL stimulus, a reduction in IκBα and an increase in p-p65, p-Akt and p-Erk were expected.

The results reflect the dynamics of the pathway activation and downstream molecular cascade in the different cell lines, with different activation rates and sustained activation times before returning to basal levels. This justifies why not every variation was observed in every condition of the two stimulation timepoints. Since we confirmed pathway activation by exogenous RANKL, next we aimed to test the effect of an inhibitory approach via RANKL blockade.

4.3. RANKL blockade by OPG-Fc

RANKL inhibition can be achieved using OPG-Fc, a recombinant protein resulting of truncated OPG fused to the Fc domain of a human IgG1[148]. OPG-Fc lacks the heparin-binding and death homology domains of OPG but has an increased circulating half-life. OPG-Fc is used in animal models of RANKL blockade since denosumab, the monoclonal antibody against RANKL, does not recognize murine RANKL.

To confirm that OPG-Fc effectively inhibits RANKL-mediated RANK activation, serum-starved MDA--MB-231 cells were incubated with hRANKL, previously incubated with OPG-Fc or not, and pathway activation was assessed by quantification of p-Erk and p-Akt by western blot (**Figure 4.4**). MAB626, a monoclonal antibody that effectively blocks RANKL[149], was used as control.

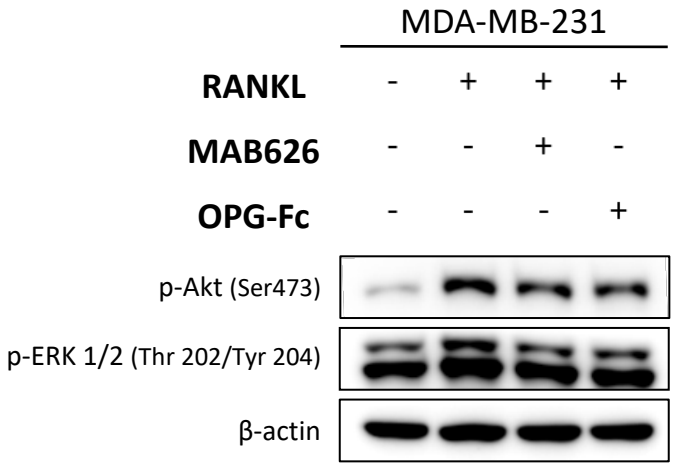

Figure 4.4 OPG-Fc has an inhibitory effect on RANKL binding to RANK. MDA-MB-231 cells were treated for 20 minutes with 1 µg/mL RANKL ± 2.5 µg/mL OPG-Fc or MAB626. Downstream targets of the RANKL/RANK pathway were analysed by western blot with β-actin as loading control.

A decrease in Akt and Erk phosphorylation was observed in cells exposed to neutralised RANKL, in comparison to RANKL stimulation, confirming that OPG-Fc impaired RANKL binding to RANK to some extent.

4.4. Effect of PARPi and PI3Ki in combination with OPG-Fc in TNBC cell viability

Since the RANKL/RANK pathway was shown to be activated by exogenous RANKL in the selected cell lines, and OPG-Fc was able to abrogate this activation, next we aimed to test the combinatory effect of RANKL blockade plus PARP and/or PI3K inhibition.

The effect of the PARPi olaparib, the PI3Ki alpelisib and OPG-Fc was assessed by measuring cell viability 72 hours after incubation with the indicated drugs (**Figure 4.5A**). Overall, the results were in line with described IC_{50} values for olaparib and alpelisib (**Table S3**), with exception of BT-20 cells treated with olaparib, which presented a higher resistance than reported. Regarding OPG-Fc, cell viability showed little variation with increasing dose, suggesting OPG-Fc alone is not able to affect cell viability.

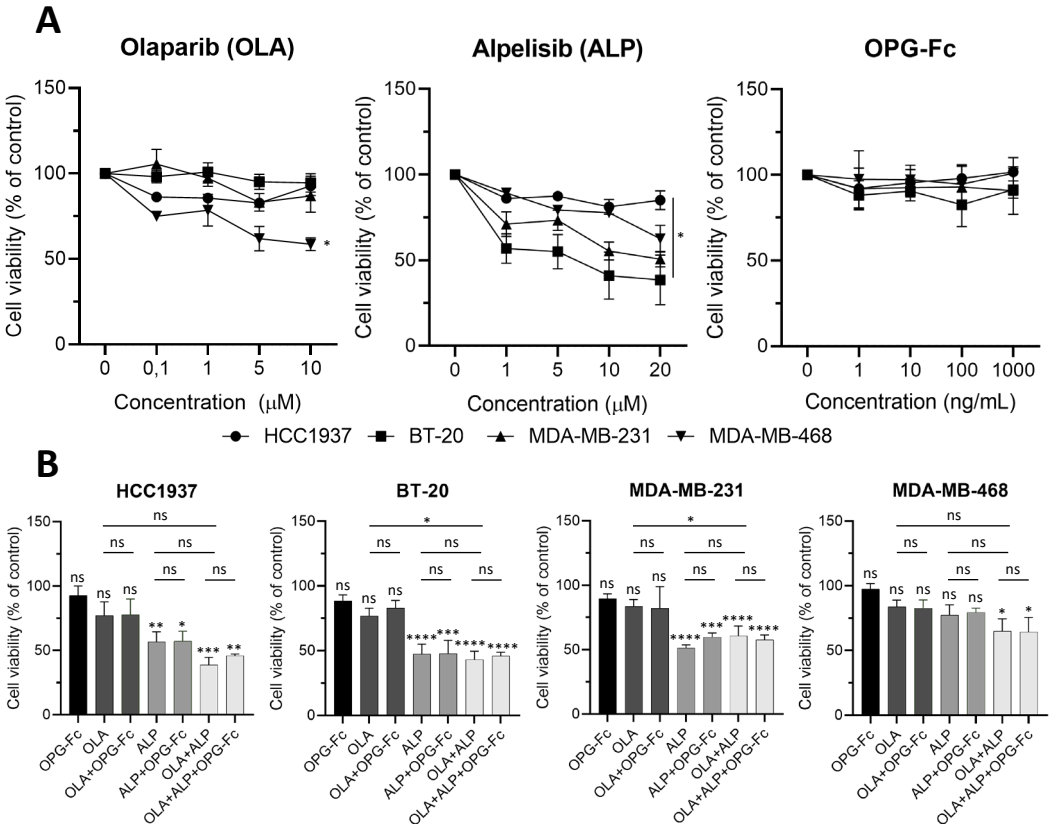

Figure 4.5 Cell viability of TNBC cells after single or combinatory treatment with olaparib, alpelisib and OPG-Fc. (**A**) 72-hour cell viability assays were conducted in HCC1937, BT-20, MDA-MB-231 and MDA-MB-468 cell lines upon treatment with different concentrations of olaparib (OLA), alpelisib (ALP) and OPG-Fc. (**B**) Cell viability was evaluated in cells treated with the indicated combinations. Results are the mean of at least three independent experiments and are presented as mean ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$; ns, not significant. One-way ANOVA test was performed using the GraphPad software.

Interestingly, the most sensitive cell line to olaparib was the BRCA-competent MDA-MB-468 cell line. Multiple factors beyond *BRCA* mutations may confer sensitivity to PARP inhibition, namely those which lead to defects in HRR[150]. For example, cell lines with wild type *BRCA* may undergo *BRCA* allelic loss, thus becoming more sensitive to PARPi[151]. Inversely, HCC1937 cells, which harbour a mutation in *BRCA1* (**Table S1**), were quite resistant to olaparib. Resistance to PARPi is a major problem in the clinical setting and can be due mainly to four mechanisms: increased drug efflux, decreased PARP trapping, restoration of HRR and restoration of stalled replication fork stability[152].

Regarding PI3K inhibition with alpelisib, the BT-20 cell line, which has two heterozygous mutations in *PIK3CA* (**Table S1**), presented the highest sensitivity to alpelisib, followed by MDA-MB-231 cells. On the other hand, MDA-MB-468 and HCC1937 were more resistant to alpelisib, in concordance with the fact that *PTEN* mutations, that both present, may confer resistance to PI3K inhibition[153]. In addition, BC cells may adopt resistance mechanisms such as increased dependency on other PI3K isoforms, e.g. cells may rapidly restore PI3K signalling through activation of p110β[154].

As previously mentioned, a synergy between PI3Ki and PARPi was described *in vitro* for BC cells[66,67]. Taking this discovery into consideration, the combinatory approaches pertain three main comparisons: olaparib versus olaparib and OPG-Fc; alpelisib versus alpelisib and OPG-Fc; and olaparib and alpelisib versus olaparib, alpelisib and OPG-Fc. However, in neither of these conditions was the addition of OPG--Fc associated with a statistically significant decrease in cell viability (**Figure 4.5B**). This indicates that there is no synergic effect between RANKL blockade with OPG-Fc and either olaparib and/or alpelisib *in vitro*.

Moreover, combination of olaparib and alpelisib in the indicated dosages did not present a statistically significant difference in cell viability compared to the drug with lowest associated viability – which was alpelisib for all cell lines. Despite this observation, HCC1937 and MDA-MB-468 cells presented a slight reduction in viability after treatment with the olaparib/alpelisib double combination.

4.5. Effect of olaparib and alpelisib on downstream molecular targets

Finally, to confirm that the reduction in cell viability was dependent of effective targeting by the drugs, namely PARP by olaparib or PI3K by alpelisib, HCC1937 and MDA-MB-468 cells were treated as before, and the specific targets analysed by western blot (**Figure 4.6**).

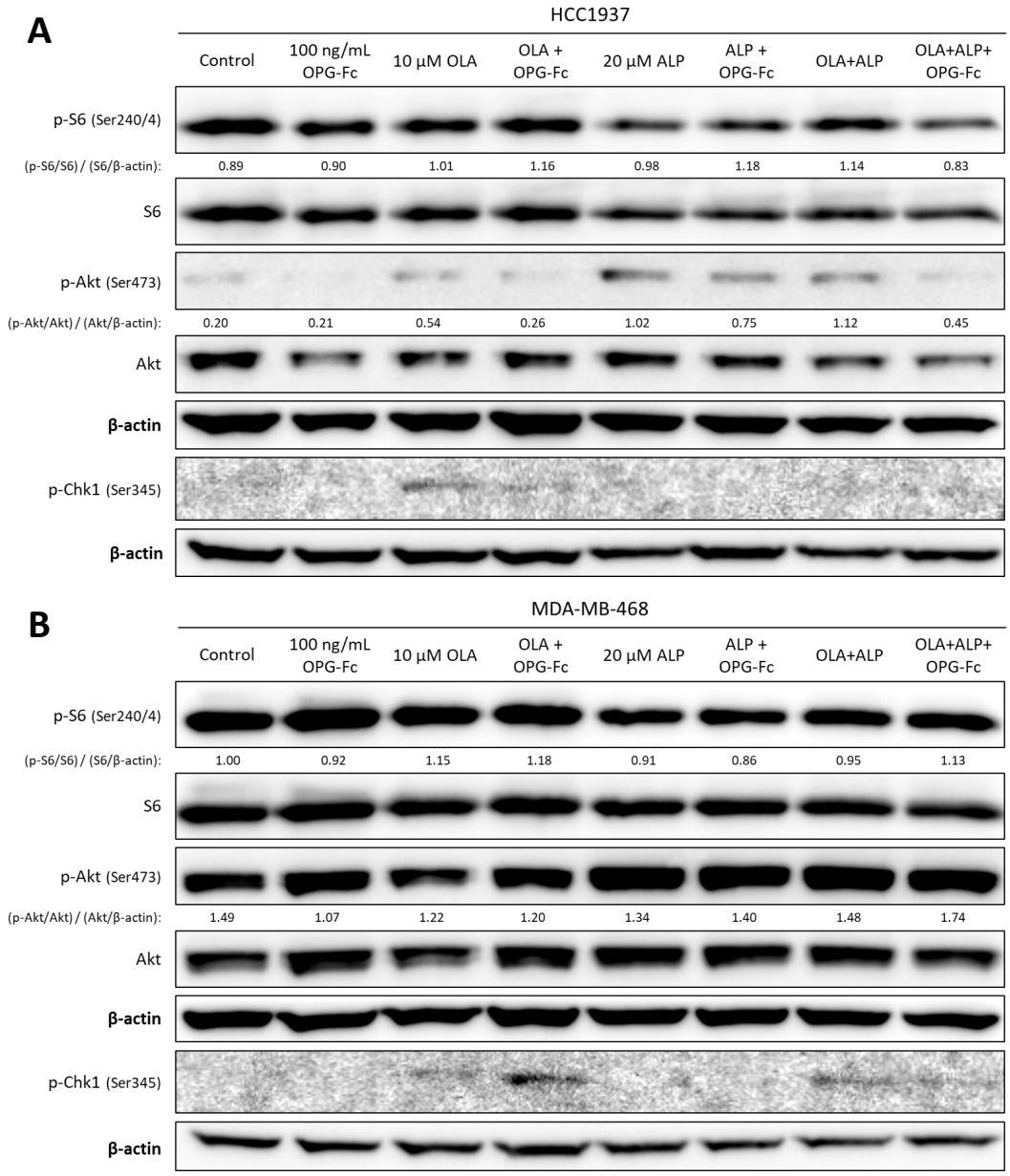

Figure 4.6 Olaparib and alpelisib affect downstream molecular targets. (**A**) HCC1937 and (**B**) MDA-MB-468 cell lines were treated with 100 ng/mL OPG-Fc, 10 µM olaparib, 20 µM alpelisib or derived combinations for 72 hours. Using β-actin as loading control, olaparib or alpelisib-related targets were evaluated via western blot. Band intensity was quantified with the ImageJ software and ratios to loading control or total protein were calculated.

Because alpelisib is a PI3Ki, it contributes to the inhibition of the PI3K/Akt/mTOR pathway, and therefore a reduction in phospho-Akt was expected following treatment. Curiously, whilst no noticeable change was seen on MDA-MB-468 cells, the opposite was observed in HCC1937 cells, i.e. presence of alpelisib promoted Akt phosphorylation. An *in vitro* 2018 work by Clement *et al.* uncovered a resistance mechanism to PI3Ki consisting of E3 ubiquitin ligase Skp2-dependent reactivation of Akt. According to this research, inhibition or depletion of PI3K could lead to increased Akt phosphorylation – an event restricted to a subset of TNBC cell lines, in which HCC1937 are included[155]. The observed increase in

p-Akt in cells treated with alpelisib is likely due to this mechanism, which means that, albeit unexpectedly, a molecular impact of alpelisib is visible. The S6 ribosomal protein is also a downstream target of the PI3K pathway, with pathway activation promoting its phosphorylation. Thus, it was also expected that upon PI3K inhibition phospho-S6 levels would diminish, although no variation in p-S6 could be observed regarding alpelisib treatment.

On the other hand, cells treated with olaparib are expected to present higher levels of phospho--checkpoint kinase 1 (Chk1). The kinase Chk1 is activated via phosphorylation downstream of Ataxia Telangiectasia and Rad3-related (ATR) following DNA damage or replication stress, arresting cell cycle progression to allow for DNA repair. Indeed, increase in p-Chk1 was observed for both HCC1937 and MDA-MB-468 cells upon olaparib treatment.

These results indicate that both olaparib and alpelisib are affecting molecular targets in TNBC cells, although to different extents, an event concomitant with their effect on cell viability. This allows us to exclude drug inefficacy as a possible explanation for the lack of synergy in the drug combinations. There are, however, limitations to this study, as analysis of downstream molecular targets was performed in a single experiment.

5. CONCLUSION AND FUTURE PERSPECTIVES

TNBC is an aggressive subtype of BC associated with poor clinical outcome. One significant constraint to the treatment of patients is the lack of targeted therapies, which would allow a more specific, and likely effective, approach. The discovery of a link between the RANKL/RANK pathway and TNBC tumorigenesis allowed for the investigation of innovative approaches to treat the disease, namely combination therapies including RANK signalling modulation.

In the present project, we hypothesised that RANKL blockade could synergise with the PI3K inhibitor alpelisib and the PARP inhibitor olaparib, broadening their applicability to include some or more TNBC patients, respectively. However, *in vitro* analyses using TNBC cell lines showed no statistically significant effect of combination regimens.

Recently, RANKL blockade was associated with higher pre-clinical efficacy of immune checkpoint inhibitors, namely in combination with PD1/PD-L1 or CTLA4 blockade[156]. As of 2020, there are two ongoing clinical trials, KEYPAD (NCT03280667) and CHARLI (NCT03161756), studying a combination of denosumab with immune checkpoint inhibitors in clear cell renal carcinoma and unresectable or metastatic melanoma, respectively. If the pre-clinical evidence is verified in these trials, the same approach should be tested on BC, since the RANKL/RANK pathway has a significant involvement in mammary tissue, both in physiological and oncological contexts. TNBC would be especially attractive in this case since it is more likely to benefit from immune checkpoint blockade than other subtypes due to its higher immunogenicity, higher PD-L1 expression and enrichment in tumour--infiltrating lymphocytes[157–159].

Since TNBC patients have very limited therapeutic options, research and development of novel molecules or even drug repurposing are of critical importance. More so considering the high heterogeneity of TNBC, which will likely require subtyping and development of personalised approaches to each subtype. Combination therapies will probably be essential for this process, and although the drug combinations proposed in this project did not show any benefit *in vitro*, RANKL blockade should be studied in depth to direct all of its potential towards the discovery of treatment alternatives for TNBC patients.

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APPENDIX

Table S1 *BRCA1/2*, *PTEN* and *PIK3CA* status of the TNBC cell lines in the panel[160,161].

Cell line	*BRCA1/2* status	*PTEN* status	*PIK3CA* status
HCC1937	*BRCA1* mutated (homozygous c.5266_5267insC)	Mutated (homozygous deletion)	Wild type
BT-20	Wild type	Wild type	Mutated (heterozygous c.1616C>G and heterozygous c.3140A>G)
MDA-MB-231	Wild type	Wild type	Wild type
MDA-MB-468	Wild type	Mutated (homozygous c.253+1G>T)	Wild type

Table S2 mRNA expression data for *RANK* (*TNFRSF11A*) and *RANKL* (*TNFSF11*) across BC cell lines, retrieved from the Cancer Cell Line Encyclopedia (CCLE)[120,162]. Values were obtained via RNASeq and are presented in Log2RPKM ordered for decreasing *RANK* expression. TNBC cells used in this project are highlighted.

Cell line	*TNFRSF11A*	*TNFSF11*
HCC70_BREAST	2.9401096	-0.46621224
AU565_BREAST	1.398290561	-13
HCC2157_BREAST	1.331681415	-13
HCC1143_BREAST	1.19790117	-5.106361658
HCC1187_BREAST	1.189016004	-4.856818136
CAL51_BREAST	1.057985114	-13
HCC1954_BREAST	0.973932394	-3.902250589
MDAMB468_BREAST	0.904624149	-13
MDAMB231_BREAST	0.838153185	-13
MDAMB175VII_BREAST	0.491170875	-4.534546938
HCC38_BREAST	0.149388964	-4.103090872
ZR751_BREAST	0.033290241	-13
HMEL_BREAST	-0.056751978	-3.674601071
BT20_BREAST	-0.0593241	-13
CAL851_BREAST	-0.233893939	-4.885490814
HCC1599_BREAST	-0.284225335	-3.534351023
SKBR3_BREAST	-0.420929371	-6.807569465
MDAMB415_BREAST	-0.498624298	-3.37132519
HMC18_BREAST	-0.853397765	-13
HCC1937_BREAST	-0.95089629	-0.796249793
EFM192A_BREAST	-1.258191943	-7.19868407
HCC1428_BREAST	-1.284746289	-13
MDAMB361_BREAST	-1.376981128	-13
HCC1419_BREAST	-1.502619572	-13
KPL1_BREAST	-1.531007627	-13

MCF7_BREAST	-1.557351949	-13
HCC202_BREAST	-1.567411078	-13
DU4475_BREAST	-1.579180223	-13
HCC1806_BREAST	-1.681640483	-5.94547517
HCC2218_BREAST	-1.705042634	-13
T47D_BREAST	-1.793258066	-13
UACC812_BREAST	-1.921447176	-13
HCC1395_BREAST	-1.973981675	-13
MDAMB436_BREAST	-2.104042041	-7.197323212
CAMA1_BREAST	-2.142396515	-13
BT549_BREAST	-2.244616152	-4.902511133
HS578T_BREAST	-2.246154962	-13
BT474_BREAST	-2.382347132	-7.05113046
HDQP1_BREAST	-2.526858607	-0.807856812
CAL120_BREAST	-2.715849158	-13
MDAMB453_BREAST	-2.779087536	-6.829299985
HCC1569_BREAST	-2.896640629	-13
UACC893_BREAST	-3.557200107	-13
JIMT1_BREAST	-3.646321172	-13
HS274T_BREAST	-3.653719341	-13
MDAMB134VI_BREAST	-3.693672779	-13
CAL148_BREAST	-4.245191587	-13
MDAMB157_BREAST	-4.918962961	-13
HS606T_BREAST	-5.098164082	-13
HS343T_BREAST	-5.374366396	-13
HCC1500_BREAST	-5.390897142	-13
HS742T_BREAST	-6.16185884	-13
HS281T_BREAST	-6.174836524	-13
HS739T_BREAST	-6.551758519	-13
BT483_BREAST	-6.691730693	-13
EFM19_BREAST	-7.179649613	-13
ZR7530_BREAST	-7.713275303	-6.134131208

Table S3 Described IC_{50} values for olaparib and alpelisib across the TNBC cell lines. Source publication is indicated next to each value.

	HCC1937	**BT-20**	**MDA-MB-231**	**MDA-MB-468**
Olaparib	96 μM[150]	7.7 μM[163]	> 90 μM[29]	5 μM[164]
Alpelisib	> 10 μM[165]	1.3 μM[166]	19.29 μM[48]	11.28 μM[48]